

PATENT SPECIFICATION

NO DRAWINGS

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COMPLETE SPECIFICATION

Diagnostic Composition and Method

We, MILES LABORATORIES, INC., a corporation organized and existing under the laws of the State of Indiana, United States of America, of 1127 Myrtle Street, Elkhart, Indiana, United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to improved diagnostic compositions and test indicators utilizing them. In particular, this invention is concerned with diagnostic test indicators useful in the qualitative detection and quantitative determination of galactose in body fluids. More particularly, this invention is concerned with diagnostic test reagent compositions and test indicators containing these test composition impregnated into bibulous carriers.

Galactose is rarely present in the urine, blood or other body fluids of normal individuals, since the galactose which is ingested by the human body is normally converted to glycogen by the liver. In the case of patients with liver impairment, however, galactose may appear in the blood-stream and urine of the patient. The occurrence of galactose in body fluids, however, is most meaningful in the cases of patients afflicted with certain metabolic disorders. Those who are unable to consist of a salt of an organic polycarboxylic to be afflicted with the metabolic disorder, galactosemia. This disease becomes evident shortly after birth and may result in physical and mental retardation and early death if not corrected. Thus, the early diagnosis and treatment of galactosemia is essential.

The presence of galactose in the blood or urine of babies indicates an abnormality in metabolism and suggests the incidence of galactosemia. It would be extremely desirable if all babies could be screened for the inci-

dence of this disease which, if not detected and treated, leads to malnutrition, cataract formation, mental retardation and finally to an early death. If detected two or three months after birth, a dietary regime can be instituted which will ensure more nearly normal development.

Previously, no adequate convenient test for galactose has been available. In those instances where galactose has been previously determined, the methods used have been cumbersome and time consuming. For example, the copper reduction test, the one most commonly used, requires the removal of glucose by treating the urine or blood specimen with either a yeast suspension or glucose oxidase. The amount of reducing substance remaining is estimated and assumed to be galactose. This is not always a valid assumption since sugars other than galactose and non-carbohydrate reducing substances may also be present.

The mucic acid and the osazone test are not reliable tests when applied to biological fluids such as blood and urine.

These tests have required the use of skilled laboratory personnel to carry out the complicated laboratory manipulations necessary to establish the presence of galactose in body fluids. An even more decided disadvantage of the tests previously available is the fact that by their very nature they were non-specific, requiring further corroborative tests to determine whether galactose or another reducing sugar was being detected. Alternatively, the separation of reducing sugars other than galactose from the fluid being tested was required.

It has now been found that galactose may be readily and accurately detected in various body fluids and other liquid solutions by means of compositions which may be used as such or preferably impregnated into a bibulous carrier such as a "dip and read" stick. These compositions include the enzyme galac-

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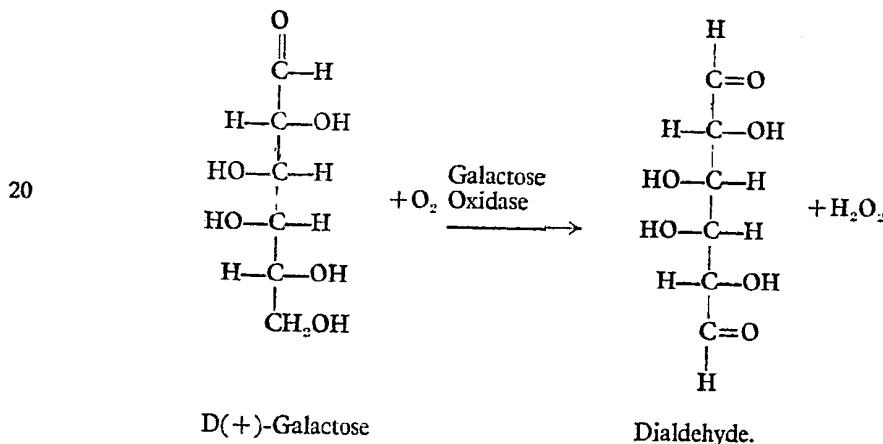
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tose oxidase. Galactose oxidase is an enzyme which catalyses the oxidation of galactose in the presence of atmospheric oxygen to produce hydrogen peroxide and an oxidation product of galactose believed to be a dialdehyde. Although galactose oxidase will effect the oxidation of galactose it has practically no effect upon glucose and other simple reducing sugars. Thus, in the case of a mixture of simple sugars, galactose oxidase will catalyse

the oxidation of galactose without any effect upon the other reducing sugars present. Galactose oxidase may be produced by various fermentation processes such as disclosed in United States of America Patent No. 3,005,714. The oxidation of galactose may be illustrated in accordance with the following equation in which a dialdehyde product is postulated:



In addition to galactose oxidase the compositions of this invention contain an indicator system which is activated upon the oxidation 25 of galactose to produce an observable phenomenon. One system which may be used uses an oxidation-reduction indicator which is oxidised by the hydrogen peroxide formed in the oxidation of galactose under the influence 30 of galactose oxidase. Few indicators, however, are oxidisable by hydrogen peroxide unless there is also present a catalyst or other oxidation promoting agent such as an electron transfer agent in whose presence the oxidation 35 of the indicator by the hydrogen peroxide readily proceeds.

For example, a second enzyme such as a peroxidase may be used to catalyse the oxidation 40 of a suitable oxidation-reducing indicator by the hydrogen peroxide formed in the oxidation of galactose. Various peroxidases are known. One which is frequently used commercially is horseradish peroxidase. Other sources of peroxidase include potatoes, fig-tree sap, turnips and white blood corpuscles. In addition to peroxidase as such, various other substances show a peroxidase-like or peroxidative activity. These substances include 45 hemin, methemoglobin, oxyhemoglobin, hemoglobin, hemochromogen, alkaline hematin and other hemin derivatives.

For the purpose of convenience the peroxidases, hemoglobin derivatives and other substances effective in promoting the oxidation 50 of oxidation-reduction indicators by hydrogen peroxide will be hereinafter referred

to as substances having peroxidative activity, although it is understood that these materials may not all function in the same way. By the term "substance having peroxidative activity" is therefore meant the above-mentioned substances as well as any other substance which is effective in promoting the oxidation of the oxidation-reduction indicator by hydrogen peroxide whether their function is one of catalyst, electron transfer agent or otherwise.

A wide variety of oxidation-reduction indicators may be used in the test indicator compositions of this invention. For example, gum guaiac, *o*-tolidine, 2,7-diaminofluorine, *o*-dianisidine, leucoindophenols and the like may be used for this purpose. The indicator preferred for the compositions of this invention is gum guaiac which has been found to give better colour development than any of the other suggested oxidation-reduction indicators.

In order to produce a test having the desired stability, reactivity and sensitivity it is essential that the aforementioned ingredients when contacted with the fluid being tested be buffered at a hydrogen ion concentration of about from pH 5.8 to pH 7.5. Preferably an approximately neutral pH should be used, for example, one in the range of about from pH 6.8 to pH 7.2. Although various buffers may be found which will maintain the pH of the ingredients within the desired range, it has been found that those buffers which properly metabolise galactose are said acid with tris-(hydroxymethyl)aminomethane produce results which, both with respect to

stability and sensitivity, are markedly superior. For example, whereas a formulation containing 0.4 M sodium citrate buffer will produce an observable colour change in a 2% solution of galactose in water in 48 seconds, a 0.4 M solution of the reaction product of glutamic acid and tris - (hydroxymethyl) - aminomethane will produce an observable colour change in about 4 seconds. Thus, the

"Tween" are Registered Trade Marks). Wetting agents are not essential, but their use contributes desired elegance to sticks made from the compositions of this invention.

5 solution of galactose in water in 48 seconds, a 0.4 M solution of the reaction product of glutamic acid and tris - (hydroxymethyl) - aminomethane will produce an observable colour change in about 4 seconds. Thus, the
 10 use of a buffer of this type gives preferred results, especially where it is desired to conduct a large number of tests in a short period of time, for example, in screening patients for galactosemia, where many tests must be run one after another. In addition to tris - (hydroxymethyl) - methylamine glutamate other polycarboxylic acid salts of tris - (hydroxymethyl) - aminomethane which may be used include tris - (hydroxymethyl) - methylamine phthalate, tris - (hydroxymethyl) - methylamine malonate and tris-(hydroxymethyl)-methylamine citrate, for example.

In formulating the compositions of this invention it has also been found desirable to use polyvinyl alcohol as a conveyor or thickening agent for the compositions. The polyvinyl alcohol has also been observed to contribute certain protective qualities to these compositions. Other thickening agents, such as gelatin, bovine serum albumin, polyvinyl pyrrolidone (PVP). Starch alginate may also be used, but polyvinyl alcohol is preferred because of its unexpected protective properties.

35 Wetting agents or surface active agents may be used in the compositions of this invention to assure an even distribution of the ingredients upon the test sticks, and, after drying, a uniform wetting of the test stick when used. Various types of wetting agents may be used for this purpose including cationic, anionic and non-ionic varieties. Examples of the wetting agents which may be used are dioctyl sodium sulphosuccinate (Aerosol OT) and polyoxyethylene sorbitan mono-oleate (Tween 81) (the words "Aerosol" and

50 Tween" are Registered Trade Marks). The compositions described above may be readily prepared by adding the polyvinyl alcohol, wetting agent if desired, material having peroxidative activity, galactose oxidase and oxidation-reduction indicator to a solution of the buffer at the desired pH. The compositions may then be used as a dip for strips or sticks or bibulous carrier material. In each instance the impregnated bibulous carrier may be dried either at room temperature or at elevated temperatures depending upon considerations of time and ease of manufacture.

55 The compositions of this invention and the methods of using them are further illustrated by the following Examples:

EXAMPLE I

A solution is made by adding to 100 ml. of 0.4 M tris - (hydroxymethyl) - methylamine-glutamate at pH 5.8, 250 mg. of bovine serum albumin, 0.5 g. potassium chloride, 2.5 ml. of 5% Tween 81 in water, 150 mg. of horseradish peroxidase, 10 ml. of o-tolidine (free base, 100 mg. in 10 ml. of 95% ethanol). The pH was readjusted from pH 6.2 to pH 5.8 with 1 N hydrochloric acid. To a 10 ml. aliquot of this mixture was then added 500 mg. of galactose oxidase assaying 5,000—8,000 units per gram. The unit of galactose oxidase activity is defined as that quantity of galactose oxidase that will give the activity equivalent to one unit of glucose oxidase as defined in D. Scott, Journal Agriculture and Food Chemistry 1, 727 (1953). Sticks of bibulous cellulose were dipped into the resulting mixture and dried at room temperature. These sticks were found to be capable of detecting the presence of 0.08% of galactose in water within 60 seconds and reacted with 2.5% galactose in urine within 3 minutes, turning from a light buff colour to blue.

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EXAMPLE II
 The following ingredients were mixed in the order indicated:

95	Tris - (hydroxymethyl) methylamine glutamate, 0.4 M (pH 7.0)	4 ml.
	Glactose Oxidase	6,000 units
	D & C Yellow No. 1	30 mg.
This mixture was then added to a mixture of:		
100	Tris - (hydroxymethyl)- methylamine glutamate, 0.4 M	2 ml.
	Peroxidase	2 mg.
There were then added to the resulting mixture:		
105	Polyvinyl alcohol, 15%	2 ml.
	Dioctyl sodium sulpho- succinate (Aerosol OT), 5%	2 ml.
	Gum guaiac, 5%	.2 ml.

Sticks were prepared as in Example I. These sticks were reactive to 0.05% galactose in water within 4 seconds and to 0.1% galactose in urine within 8 seconds.

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EXAMPLE III

The procedure of Example II was followed except that in place of tris-(hydroxymethyl)-methylamine-glutamate there was used a 0.4 M solution of tris - (hydroxymethyl)-methylamine malonate. The sticks containing the resulting formulation were found to be capable of detecting 0.50% of galactose in water within 44 seconds.

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The procedure of Example II was followed except that for the buffer there was used a 0.4 M solution of tris - (hydroxymethyl)-methylamine phthalate. The sticks containing the resulting formulation were found to be capable of detecting 0.05% of galactose in water within 8 seconds.

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EXAMPLE IV

The procedure of Example II was followed except that the buffer used was a 0.4 M solution of tris - (hydroxymethyl) - methylamine citrate. The sticks containing the resulting formulation were found to be capable of detecting 0.05% of galactose in water within 48 seconds.

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The above Examples illustrate that various compositions prepared in accordance with this invention may be used for the detection of

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Galactose Oxidase
Substance having peroxidative activity
Polyvinyl alcohol
Oxidation-reduction indicator
Buffer (pH 6.8-7.2)
Wetting agent (if used)

galactose in fluids including urine. Test sticks impregnated with these compositions may be used to give a quantitative estimate of the galactose present in the fluid being tested by comparing the colour produced upon dipping the stick into the test fluid with a previously prepared colour chart at a fixed time after dipping.

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Although the invention has been described with respect to the use of sticks of bibulous cellulose, it is also anticipated that the various reagents can be selectively adsorbed on to other forms of cellulose including cellulose ion exchangers. The use of the reagents in solution is also included, for example, for use in the testing of urine-wet diapers. Similarly, the ingredients may be combined into tablets or fibres used as bibulous carriers for the reagents.

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Although in general it is unnecessary to pretreat an urine or salt solution suspected of containing galactose in order to remove ions which may interfere with the test for galactose, it may sometimes be desirable to do so. For this purpose, the urine or other solution may be contacted with an ion exchange resin previous to testing with the diagnostic compositions of this invention.

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The concentrations of the various reagents used in this invention may be varied widely to suit the circumstances of use. In general, however, the following operable ranges given in terms of 100 millilitres of solution may be considered preferred ranges of concentration:

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Galactose Oxidase	10,000 to 30,000 units
Substance having peroxidative activity	1 to 10 mg.
Polyvinyl alcohol	1 to 3 g.
Oxidation-reduction indicator	10 to 50 mg.
Buffer (pH 6.8-7.2)	0.2 to 1 M
Wetting agent (if used)	0.1 to 0.5 g.

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In summary, this invention provides compositions for the detection of galactose in fluids, especially body fluids such as urine. The compositions include galactose oxidase, a substance having peroxidative activity, an oxidation-reduction indicator, a buffer which is a salt of a polycarboxylic acid with tris-(hydroxymethyl)-aminomethane and, preferably, polyvinyl alcohol. These compositions may also include other thickening agents such as gelatin or albumin and wetting agents as well as other ingredients desired to contribute elegance to the compositions. These various compositions may be used in various forms such as in solutions, tablets, fibres and absorbent columns. An especially preferred test indicator incorporates the diagnostic composition as an impregnation upon a stick or strip of bibulous cellulose.

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tion of galactose in fluids which comprises galactose oxidase, a substance having peroxidative activity, an indicator which changes colour in the presence of the substance having peroxidative activity and a reaction product resulting from the interaction of galactose with oxygen in the presence of the galactose oxidase and, as a buffer, a polycarboxylic acid salt of tris - (hydroxymethyl)aminomethane, the buffer and the amount used thereof being effective to maintain the composition at a pH of from pH 5.8 to pH 7.5 when contacted with the fluid being tested.

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2. A composition as claimed in claim 1, wherein the buffer, and the amount used thereof is effective to maintain the composition at a pH of from 6.8 to 7.2 when contacted with the fluid being tested.

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3. A composition as claimed in claim 1 or 2, wherein the substance having peroxidative activity is a peroxidase.

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4. A composition as claimed in claim 1

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WHAT WE CLAIM IS:—

1. A diagnostic composition for the detec-

or 2, wherein the substance having peroxidative activity is horseradish peroxidase. 30

5. A composition as claimed in claim 1 or 2, wherein the substance having peroxidative activity is hemin, methoglobin, oxyhemoglobin, hemoglobin, hemochromogen or hematin. 35

6. A composition as claimed in any of claims 1 to 5, wherein the buffer is tris-(hydroxymethyl)-methylamine glutamate. 40

7. A composition as claimed in any of claims 1 to 5, wherein the buffer is tris-(hydroxymethyl)-methylamine phthalate. 45

8. A composition as claimed in any of claims 1 to 5, wherein the buffer is tris-(hydroxymethyl)-methylamine malonate. 50

9. A composition as claimed in any of claims 1 to 8, wherein the indicator is gum guaiac.

10. A composition as claimed in any of claims 1 to 8, wherein the indicator is *o*-toluidine.

11. A diagnostic composition for the detection of galactose in fluids substantially as described with reference to any of the Examples.

12. A process for detecting galactose in fluids which comprises contacting the fluid to be tested with a composition as claimed in claim 1.

13. A test indicator for the detection of galactose in fluids comprising a bibulous carrier impregnated with galactose oxidase, a substance having peroxidative activity, an indicator which changes colour in the presence of substance having peroxidative activity and a reaction product resulting from the interaction of galactose with oxygen in the presence of said galactose oxidase, polyvinyl alcohol, and as a buffer, a polycarboxylic acid salt of tris-(hydroxymethyl)-aminomethane, the buffer and the amount used thereof being effective to maintain the composition at a pH of from pH 5.8 to pH 7.5 when contacted with the fluid being tested.

14. A test indicator as claimed in claim 13, wherein the bibulous carrier is bibulous cellulose.

15. A test indicator as claimed in claim 13 or 14, wherein the buffer and the amount used thereof is effective to maintain the composition at a pH of from 6.8 to 7.2 when contacted with the fluid being tested.

ELKINGTON AND FIFE,
Chartered Patent Agents,
High Holborn House, 52-54 High Holborn,
London, W.C.1,
Agents for the Applicants.

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